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Characterization of *Toxoplasma gondii C*alcium regulator proteins TGGT1_253640 and TGGT1_222060 for *Toxoplasma gondii* growth and invasion

By

Abby Delapenha

Thesis

Submitted in partial fulfillment of the requirements for Honors in Biology at the University of Mary Washington Fredericksburg, Virginia

04/23/2023

This Thesis by Abigail Delapenha is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

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Biography

Abby Delapenha is from Bristow, Virgina and graduated from Osbourn Park Senior High School (Manassas, Virginia) in 2019. At Osbourn Park High School, she was a part of the Biotechnology Program and had the opportunity to take science focused courses. During her junior and senior year of high school, she was student at Governor's School at Innovation Park in Manassas, Virginia. At the Governor's School, she was given the opportunity to conduct her own research. This experience created a passion for research. She is a member of the Honors Program, Jepson Scholars Program, President of the Honor Council, Vice president of Chi Beta Phi, and a member of Phi Eta Sigma and Phi Beta Kappa. She has been on the Dean's (Spring 2020, Spring 2021, and Fall 2021) and President's List (Fall 2019, Fall 2020, Spring 2022, and Fall 2022). She started her research journey at UMW during summer 2019 through the Jepson Scholars program. Her research focused on a plant pathogen, phytoplasma, in the spotted lanternfly. She presented her research during the Fall 2019 semester at NURVA. During the Spring 2021 semester, she did URES in research on apoptosis in *Crithidia fasciculata.* She continued her research during Summer Science 2021 and presented her work at the Summer Science Symposium. Her current research focuses on calcium regulation in *Toxoplasma gondii*. She has presented her research at the American Society of Cellular Biology (ASCB), and the Association of Southeastern Biologists (ASB). Her research has helped her learn more about disease processes in various organisms, information that will help her as she studies medicine at the George Washington School of Health and Medical Sciences.

Acknowledgements

I would like to thank Dr. Swati Agrawal for her mentorship throughout this project and Emily Sizemore for her assistance on this project. I would also like to thank Dr. Abbie Tomba and Dr. Thresa Grana for their advice throughout this project.

Abstract

Toxoplasma gondii is an opportunistic apicomplexan parasite infecting humans and livestock. Infection in immunocompromised individuals can cause neurological damage and infection during pregnancy can lead to fetal death. These parasites engage in a complex life cycle, involving repeated invasion of the host cell and egress from the host cell. Calcium signaling is an important regulatory mechanism for many essential processes in the parasite, including gliding motility (actomyosin-dependent mode of motion), invasion, and egress. Our work here focuses on two previously uncharacterized calcium regulator proteins (TGGT1_253640 and TGGT1_222060). To characterize the role of these proteins in parasite viability and calcium regulation, we fused an auxin degron system using CRISPR-cas9 gene modification technique. We have localized the TGGT1_253640 to endoplasmic reticulum of the parasite. The protein is downregulated upon the addition of auxin as early as four hours thus providing a tool to study the function of the protein. Future work aims to determine the role of these proteins in parasite growth and fitness using growth and replication assays.

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Introduction

Toxoplasma gondii (*T. gondii*), the causative agent of toxoplasmosis, is an apicomplexan parasite that infects humans and livestock. These parasites cause toxoplasmosis infection through their complex lytic cycle. The steps for the lytic cycle include host cell attachment, host cell invasion, intracellular replication, and host cell egress (Arrizabalaga and Boothroyd) (Fig.1). Toxoplasmosis is defined by the CDC as a Neglected Tropical Disease (NTD) (2022). NTDs affect the world's poorest people and cause decreased workplace productivity, trapping individuals in a cycle of poverty and disease (CDC, 2022). About one third of the global population is infected with *T. gondii*. Most individuals infected with *T. gondii* experience mild symptoms or are asymptomatic (Attias et al., 2020). Although for most individuals' toxoplasmosis infection is harmless, infection can cause health complications in certain groups of people. In immunocompromised individuals, (individuals with AIDS, individuals undergoing organ transplantation, or individuals undergoing chemotherapy), infections can cause neurological damage. Additionally, infection by toxoplasma during pregnancy can lead to fetal death (Attias et al., 2020). Treatments used against *T. gondii* infection have high rates of failure and are unable to treat toxoplasmosis once it has entered its latent stage (Dunay et al., 2018). In the latent stage, the parasite encysts in various organs, including the brain, retina, cardiac muscles, and skeletal muscles. Reactivation of latent stage parasites is common in immunocompromised individuals and, if untreated, can cause significant morbidity and 100% mortality (Dunay et al., 2018). In addition to high failure rates, treatments for toxoplasmosis have been found to have adverse outcomes (Shammaa et al., 2021). The adverse outcomes of the treatment methods were assessed using the FDA Adverse Event Reporting System (FAERS, a database that contains information submitted to the FDA involving "adverse event reports, medication error reports and product quality complaints") (FDA, 2018). The gold standard

treatment for toxoplasmosis is a combination of pyrimethamine and sulfadiazine (Dunay et al., 2018). Pyrimethamine is reported to cause the most adverse outcomes, followed by sulfadiazine (Shammaa et al., 2021). In comparison to all toxoplasmosis treatments, most death and serious outcome reports ("hospitalization, life-threatening, disability, congenital anomaly and/or other serious outcomes") were due to treatment by Pyrimethamine (FDA, 2015). The ineffectiveness and adverse outcomes of the existing treatment options for toxoplasmosis highlight the importance of finding alternative drug pathways that will efficiently and safely treat toxoplasmosis.

More effective treatments targeting tissue entry and egress for chronic and latent stages can be developed, by studying mechanisms that contribute to parasite fitness. Calcium is used in the regulation of many essential processes in *T. gondii* including gliding motility (actomyosin-dependent mode of motion), invasion, and egress. The concentration gradient difference (~20,000-fold difference) between *T. gondii's* intracellular and extracellular environment (higher calcium concentration) provides the signal that regulates the parasite's essential processes. The increase of calcium in the parasite's cytoplasm due to calcium's entry; is the start of calcium signaling (Triana et al., 2018). It is important for *T. gondii* to maintain a basal calcium concentration (70-100 nM), as too much calcium can be deadly. Calcium levels in *T. gondii* are maintained by calcium ATPases, channels, kinases, second messengers (IP3 and cADP ribose), and calcium binding proteins (calmodulins and calmodulin-like proteins (CaBPs)) (Calixto, 2022). The exact mechanism of how second messengers, IP3 and cADP ribose, are involved in signaling is not known because there are no second messenger receptors in *T. gondii* (Triana et al., 2018). It is predicted that IP3 and cADP ribose stimulate the release of calcium from microsomes (Triana et al., 2018). The release of calcium by IP3 is inhibited by the IP3 receptor

inhibitor xestospongin C (Triana et al., 2018). Calcium dependent protein kinases (CDPKs) lead to the phosphorylation of proteins involved in the microneme exocytosis and the activation of the actomyosin system (Dubois, 2019). CDPKs are activated by intracellular calcium release in response to IP3 (Dubois, 2019). Regarding calcium binding proteins, the EF-hand is a common motif that binds calcium and activates transduction pathways. There have been 68 EF hands identified in the *T. gondii* genome.

This study focused on two previously unstudied genes (TGGT1_253640 and TGGT1_222060) which are putative calcium transporters whose regulation and function is unknown. These two genes were chosen because of they have a calcium transport domain. This study characterized the function of TGGT1_253640 and TGGT1_222060 by endogenously labeling the protein with an auxin-inducible degron (Brown et al., 2018). This novel genetic editing system allowed for conditional knockdown of proteins allowing a temporal characterization of the protein function during various parasite life cycle stages such as invasion, replication, and egress. In the initial stages of the study, the mAID system and CRISPR cas-9 system were used to facilitate the conditional knockdown of the proteins (Fig. 2). The CRISPR cas-9 system was used to tag the C-terminus of the gene of interest (GOI) and allow for the integration of the mAID cassette with an HA tag (tags the POI), which binds the auxin and provides the signal for the GOI to be degraded (Fig. 3). In the secondary stages, transgenic parasites were evaluated for their fitness (in comparison to wild type) and invasion and egress competency. Finally, a phylogenetic evaluation of the *T. gondii* protein with other apicomplexan parasites such as *Plasmodium falciparum* and *Cryptosporidium parvum* will allow characterization of protein domains that are particularly suitable for developing drug targets.

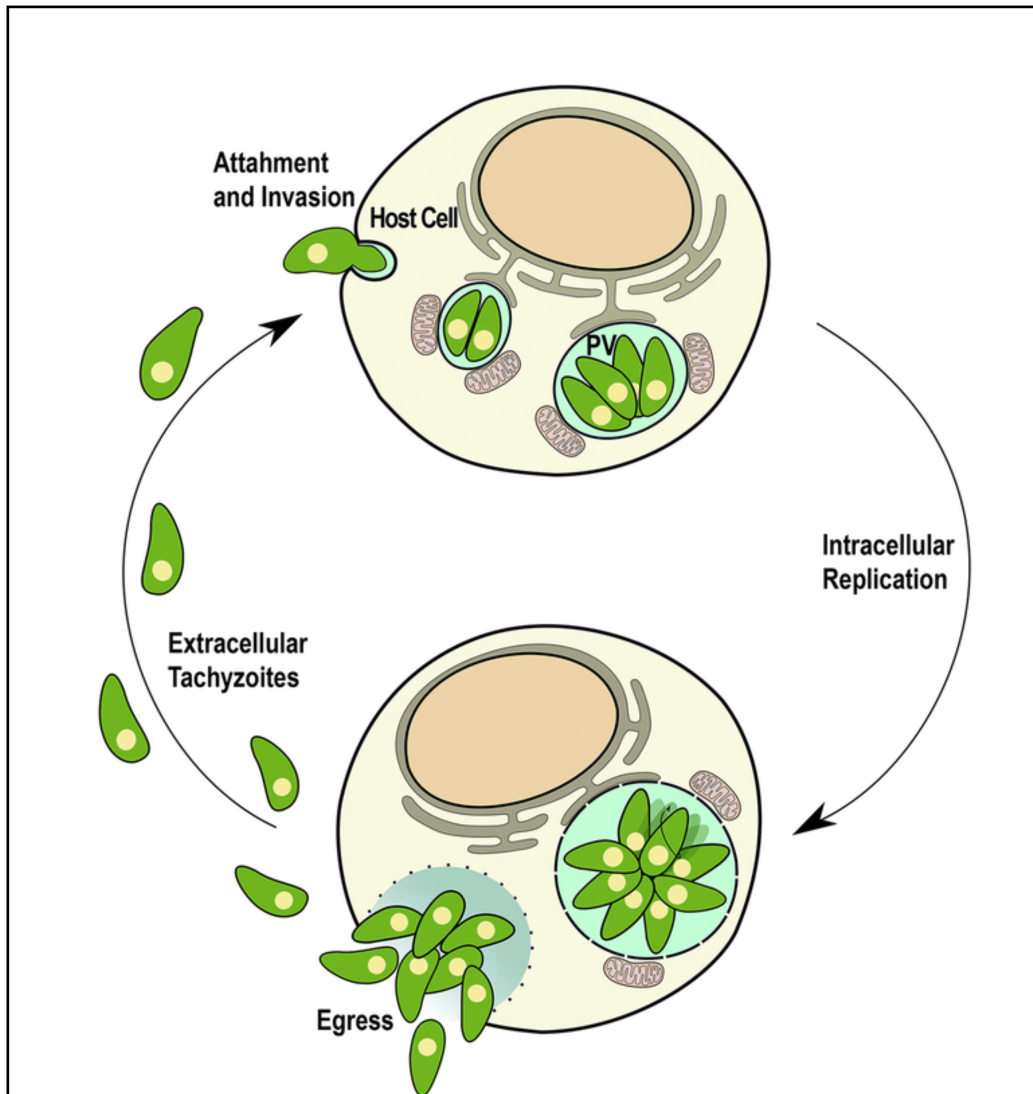


Figure 1: Lytic Cycle of *T. gondii* (Triana et al., 2018).

Rationale

Calcium signaling is important for the proper functioning of *T. gondii*. Changes in the intracellular calcium levels are responsible for triggering features of the parasite's lytic cycle including secretion of proteins essential for host cell attachment, motility, invasion, and egress (Márquez-Nogueras et al., 2021). Parasite invasion consists of the following steps: "gliding motility, conoid extrusion, secretion of specific proteins, attachment to the host cell, and active invasion" (Pace et al., 2014). These steps are all initiated by changing intracellular calcium levels either from Calcium entering the cytoplasm or being released from intracellular calcium stores

(Pace et al., 2021). The lytic cycle is how the parasite reproduces and causes infection in the host cell; thus, proper calcium signaling contributes to the pathogenicity of *T. gondii.* By performing a conditional knockdown of TGGT1_253640 and TGGT1_222060, the specific role of calcium regulation by these two Proteins of Interest (POIs) will be determined. Knowing the role of these POIs may allow for the development of more effective treatments that target the processes of calcium regulation in the parasite.

Calcium compartments in *T. gondii* include the endoplasmic reticulum (ER) and Golgi apparatus. Additionally, acidocalcisomes and endosome-like compartments (vacuole) could have a role in calcium signaling (Triana et al., 2018). Acidocalcisomes store calcium and contain a plasma membrane-type calcium-ATPase, TgA1, that is used for uptake of calcium (Triana et al., 2018). The presence of a plant like vacuole (PLV) has been characterized in *T. gondii*. This PLV has also been shown to store calcium; exposure of *T. gondii* to glycyl-L-phenylalanine-naphthylamide (GPN), a dipeptide that stimulates calcium release, resulted in an increase in cytosolic calcium levels (Triana et al., 2018). This demonstrates the presence of calcium in the PLV. In addition to these calcium stores, possible transient receptor potential (TRP) channels have been found in *T. gondii*, but they have not yet been characterized. TRP channels localize to the plasma membrane, where calcium influxes into *T. gondii's* cytoplasm or intracellular stores.

The major calcium store in *T. gondii* is the Endoplasmic reticulum. Calcium is constitutively effluxed from the ER (the mechanism is unknown) and pumped back into the ER via a SERCA-type calcium ATPase (Vella et al., 2021) This pump serves to counteract the constitutive ER calcium efflux. The calcium efflux from the ER is to ensure that the ER is not overloaded with calcium (Triana et al., 2018). When SERCA is blocked using thapsigargin, there

is a rise in cytosolic calcium levels, because the SERCA is unable to pump the calcium back into the ER (Vella et al., 2021).

In a study performed by Li et al. (2021), downregulation of TgTPC (a protein a part of the Two-pore channel (TPC) family) in calcium regulation of Apicomplexa showed reduction in parasite fitness. The downregulation of TgTPC led to a reduction in the number of parasites per Parasitophorous Vacuole (PV), prolonged egress, and a reduction in invasion. All these factors are mechanisms that help *T. gondii* infect the host cell. This study shows that when calcium channels are unable to provide the parasite with calcium, the ability of the parasite to infect the host is negatively affected.

In addition to the study conducted by Li et al. (2021), a study done by Márquez-Nogueras et al. (2021) looked at the importance of calcium regulation in *T. gondii*. The project focused on TgTRPPL-2, a protein proposed to form a TRP calcium channel, in *T. gondii*. Through tagging the 3' locus of TgTRPPL-2 and the use of an immunofluorescence analysis, it was determined that TgTRPPL-2 localizes to the Endoplasmic Reticulum (ER), the site of calcium storage, and the plasma membrane. TgTRPPL-2 was found to have a role in the influx of calcium to the plasma membrane. The influx of calcium into the plasma membrane allows the calcium dependent events, which facilitate the pathogenicity of *T. gondii*, to occur.

The physiological role of TgTRPPL was determined using cas-9 to generate TgRPPL mutants. Plaque assays, which show the invasion, replication, and egress cycle of parasites, determined that TgTRPPL mutants had limited growth. The TgTRPPL-2 mutant plaques were smaller than those of the non-mutated TgTRPPL. By complementing the TgTRPPL-2 mutant with Cosmid PSBLZ13 (which contains the whole genomic locus of the TgTRPPL-2), normal parasite growth was restored. This indicates the importance of calcium regulation for normal

parasite growth. When using TRP channel inhibitors, TgTRPPL-2 was inhibited. This inhibition of the TgTRPPL resulted in decreased parasite growth. This study highlights the important role calcium regulators play in *T. gondii's* growth, lytic cycle, and calcium levels.

These two studies indicate the importance of calcium regulation in *T. gondii* and provide the basis for further research into *T. gondii's* calcium regulator proteins. TgTPC and TgTRPPL-2 represent two of the many hypothesized calcium regulator proteins in *T. gondii*. By studying other calcium regulator proteins, more can be learned about the calcium pathways in *T. gondii* and develop drugs that target specific regulator proteins. Because of the negative effects on *T. gondii's* fitness and ability to infect the host cell upon downregulation of TgTPC and TgTRPPL, it is expected that the conditional knockdown of TGGT1_253640 and TGGT1_222060 will lead to decreased growth and invasion of *T. gondii,* causing a decrease in pathogenicity.

Preliminary Results

Generation of auxin- inducible degron (AID) conditional knockdown cells lines

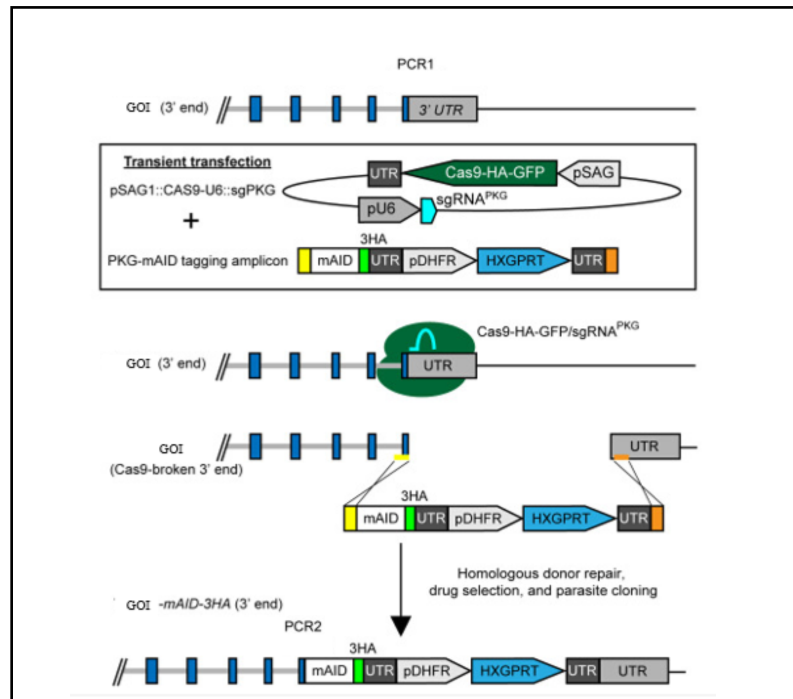
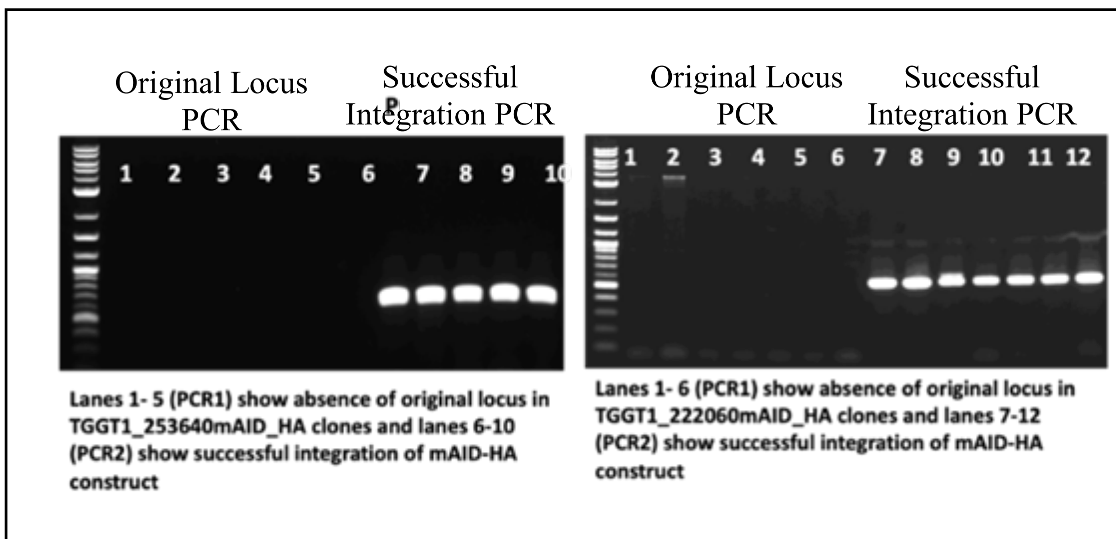


Figure 2: Auxin- Degron Conditional Knockdown system for TGGT1_235640 and TGGT1_222060 (Brown et al., 2018).

The mAID system is an inducible system used for the conditional knockdown of proteins (Brown et al., 2018). This system can be used to study the function of essential genes in *Toxoplasma gondii.* Proteins of interest (POI) can be tagged with an AID (Brown et al., 2018). When parasites are treated with auxin, a Skp-CullenF Box (SCFTIR1) is activated. This complex bind and degrades POIs tagged with AID. This system was used to generate conditional mutants for TGGT1_253640 and TGGT1_222060 genes in *T. gondii* parasites. Fig. 2 shows a schematic for the constructs used for generation of these parasite lines. The mAID cassette recognizes the presence of auxin in the parasite and signals for the degradation of the POI. The HA tag is used to tag the POI. The HXGPRT construct is used to select for parasites that have successfully integrated the mAID-HA cassette.



PCR Screens for Transgenic Parasites

Figure 3: PCR Screens for transgenic parasite clones for TGGT1_253640 and TGGT1_222060. Each number represents a distinct clone (Agrawal et al., unpublished).

Drug selection was used to select for a (m)AID-3HA, Floxed HXGPRT construct. Parasites were exposed to mycophenolic acid (25 μg/ml) supplemented with xanthine (50 μg/ml)

after 24 hours of drug selection. A screen PCR was conducted on the parasite genomic DNA to determine whether or not the tagging was successful. Fig. 3 shows the desired modification of the respective gene loci for each of the proteins being studied. PCR 1 shows loss of original locus and PCR2 shows proper integration of the mAID-HA cassette for each of the genes.

Materials and Methods

To analyze the effects of the conditional knockdown of the two POIs, the transgenic parasites were assessed for growth defects and localization of proteins.

Parasite and host cell manipulation and Auxin downregulation

HFF (Human Foreskin Fibroblast) cells (host cells) and *T. gondii* parasites were cultured in a 37 °C 5% CO_2 incubator in DMEM medium for regular maintenance. For passing parasites, extracellular parasites were passed every 2-3 days on fresh confluent HFF cells. For downregulation of proteins, the mAID system protocol established by Brown et al. (2018) was used. Briefly 100 μM concentration of auxin was used to turn off the POI tagged with the mAID cassette. Parasites were incubated for 4-6 hours in both treatments until protein knockdown was observed through immunofluorescence analysis.

Immunofluorescence analysis and Western blot

Immunofluorescence analysis and western blot were used to test for successful downregulation of the POI and followed the procedure as established by Agrawal et al. (2009). More detailed methods can be found in the referenced paper. For immunofluorescence analysis, human fibroblasts were infected with the appropriate parasite strain, fixed 24 hours after infection with 3% paraformaldehyde, and permeabilized with 0.25% Triton X-100 in PBS. Primary antibodies against rabbit anti-HA (1:100 to 1:500 dilution) will be used to localize proteins of interest. Stained cells were analyzed using an epifluorescence microscope For western blotting analysis protein samples from auxin induced and uninduced parasites were loaded onto precast SDS-PAGE gels. After electrophoresis, proteins were transferred to a

nitrocellulose membrane. Blots were probed with antibodies against anti-HA (1:1000; clone Poly9023, BioLegend) antibody. Horseradish peroxidase-conjugated anti-mouse was used to detect proteins of interest.

Plaque Assay and Real-Time Growth Assay

To determine whether the POIs are essential, a plaque assay was conducted. The plaque assay protocol was followed as established by Márquez-Nogueras et al. (2021). More detailed methods can be found in the referenced paper. Egressed tachyzoites infected confluent HFF cells in six well plates for 7 days. The resulting clearings, plaques, represent the invasion/egress cycle of *T.gondii*. For this procedure a parental cell line, TIR (no mAID-HA cassette) served as the control and TGGT1_253640, which has integrated the mAID-HA cassette, served as the experimental group. For each cell line, one set of plates was incubated with Auxin to turn off the POI and a control set was incubated without Auxin. After incubation, the cells were fixed with ethanol and stained with crystal violet. The comparison of parental versus transgenic plaques, accounts for any changes in parasite growth or fitness as a result of the mAID cassette addition.

Results

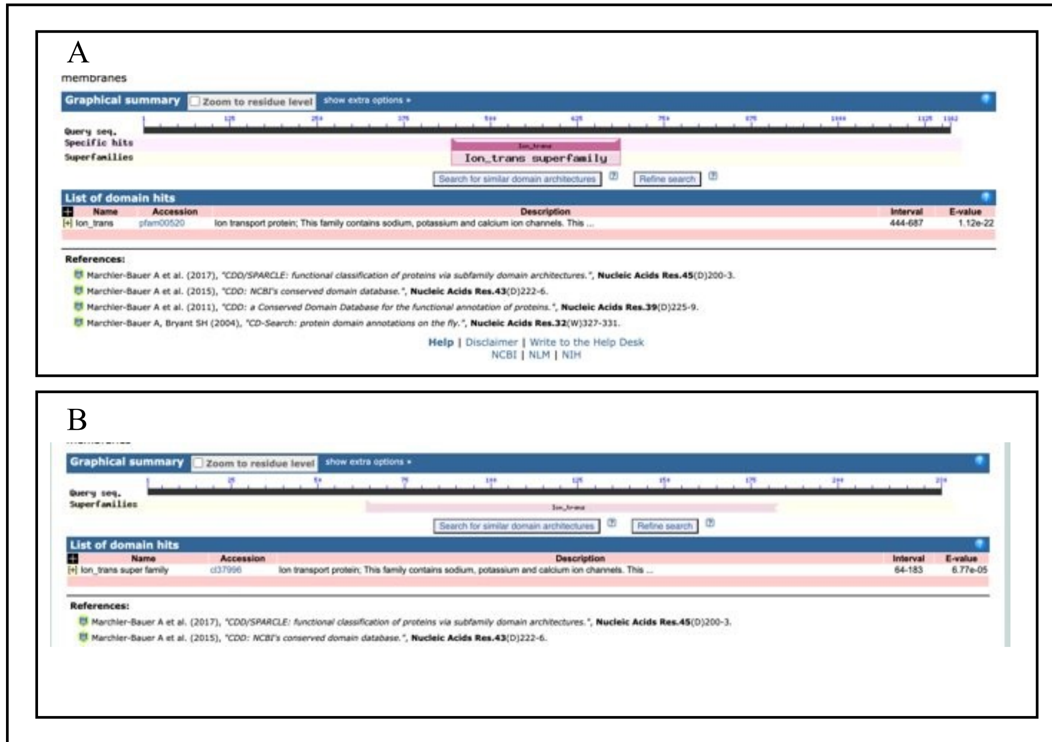
TGGT1_253640 co-localizes to the Endoplasmic Reticulum of *T. gondii*

Through BLAST domain analysis, it was determined that TGGT1_253640 contains an ion transport protein with a domain for cation transport (Fig. 4). BLAST domain is a bioinformatics toolkit that can be used to determine protein homology and putative function of proteins. Based on BLAST analysis, it was determined that the TGGT1_222060 that the cation transport domain is found in between amino acids 419 and 699 within the protein. The TGGT1_253640 protein is 178 amino acids long and the cation transport domain is found in between amino acids 29 and 207.

TGGT1_253640 also has been determined to have at least 3 transmembrane domains through Potter visualization and TMHHM analysis. Protter is a protein visualization software

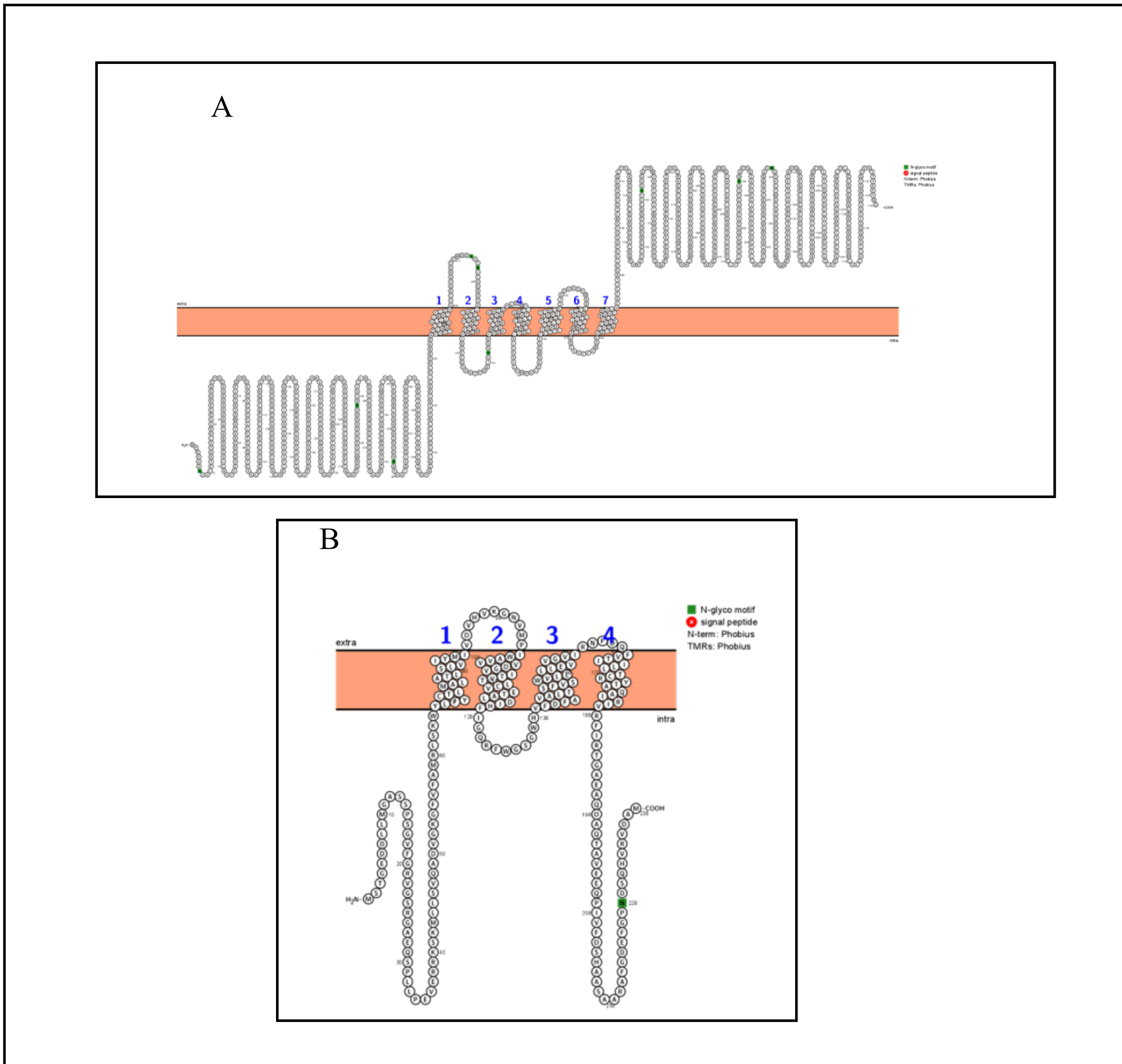
that can be used to determine if a Protein of Interest (POI) has membrane association (Fig. 5). It can be used to determine the orientation and number of transmembrane domains of a POI. Protter predicts 4 Transmembrane helices for TGGT1_253640. The program also shows that the N-terminus and C-terminus of TGGT1_253640 are intracellular. TMHHM analysis is used to determine the number of transmembrane domains of a protein embedded in the cellular membrane (fig 6). It predicts the presence of three transmembrane domains in TGGT1_253640. In TGGT1_222060, Protter predicts the presence of 7 Transmembrane helices, an intracellular N-terminus and an extracellular C-terminus.

An Immunofluorescence Assay (IFA) of intracellular parasites was conducted to determine the location of TGGT1_253640 (Fig. 7). Co-localization of TGGT1_253640 (anti-HA stain) with the sarco-endoplasmic reticulum $Ca2^{+}$ ATPase (TgSerca) (ER marker) was seen in the IFA (Nagamune et al., 2007). This supports the conclusion that there is partial co-localization of TGGT1_253640 to the Endoplasmic Reticulum, which is a major store of calcium in *T. gondii*. The partial localization to the Endoplasmic reticulum indicates that TGGT1_25360 may localize to multiple organelles in the secretory pathway in *T. gondii*. Further localization experiments using other organellar markers and transmission electron microscopy need to be conducted to determine the full localization of TGGT1_253640.



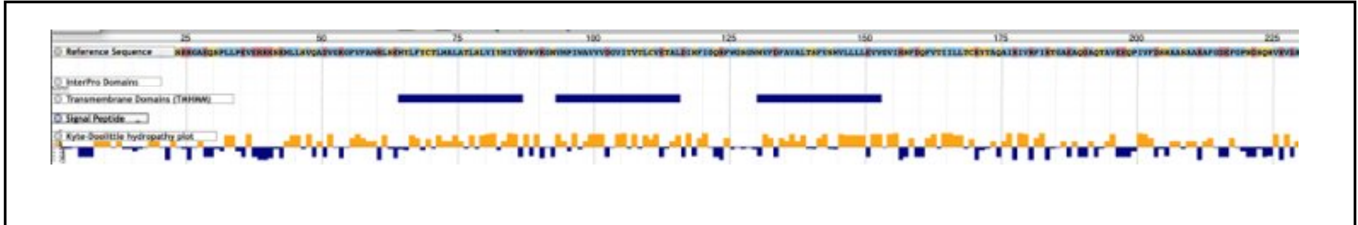
BLAST analysis for putative function prediction of TGGT1_222060 (A) and TGGT1_253640 (B) proteins.

Figure 4: BLAST analysis for putative function prediction of TGGT1_222060 (A) and TGGT1_253640 (B) proteins. BLAST analysis of both proteins indicates a distinct domain for cation transport function. The high e-value for both proteins indicates a high probability of the predicted function.

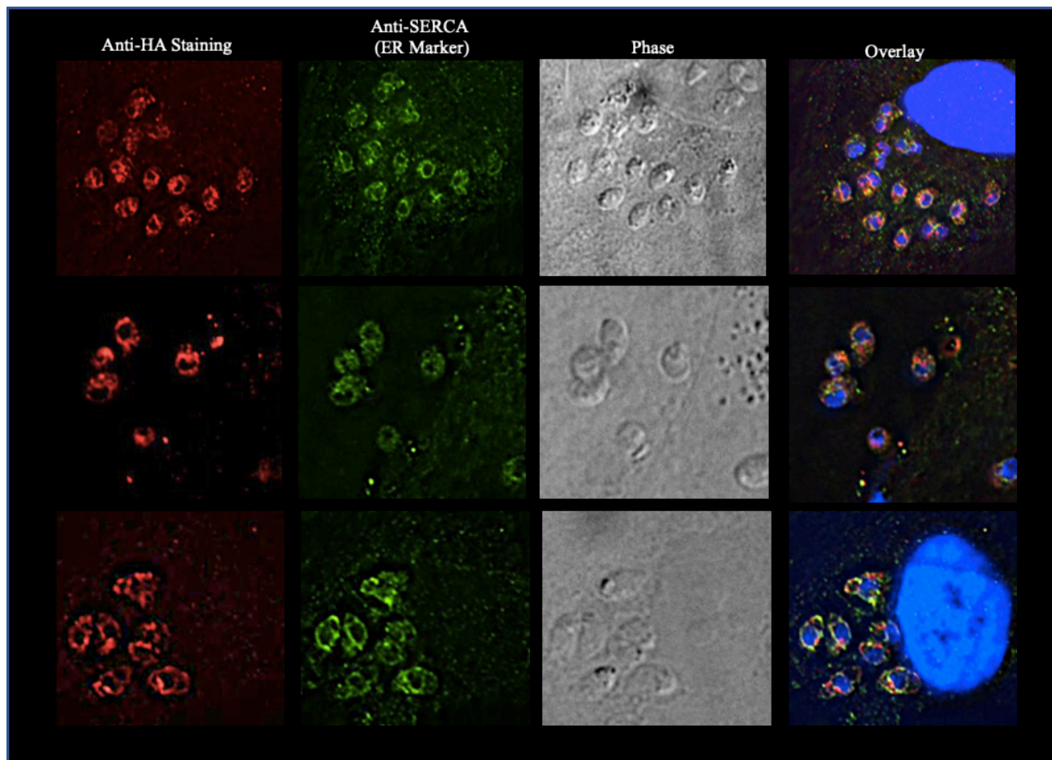


Protter visualization transmembrane topology prediction of TGGT1_222060 and TGGT1_253640 proteins.

Figure 5: Protter visualization transmembrane topology prediction of TGGT1_222060 and TGGT1_253640 proteins. Protter visualization program was used to predict TGGT1_222060 (A) and TGGT1_253640 (B) protein orientation.



TMHMM transmembrane topology prediction of TGGT1_253640
Figure 6: TMHMM analysis of the protein TGGT1_253640. TMHMM analysis was used to predict the number and location of transmembrane domains.



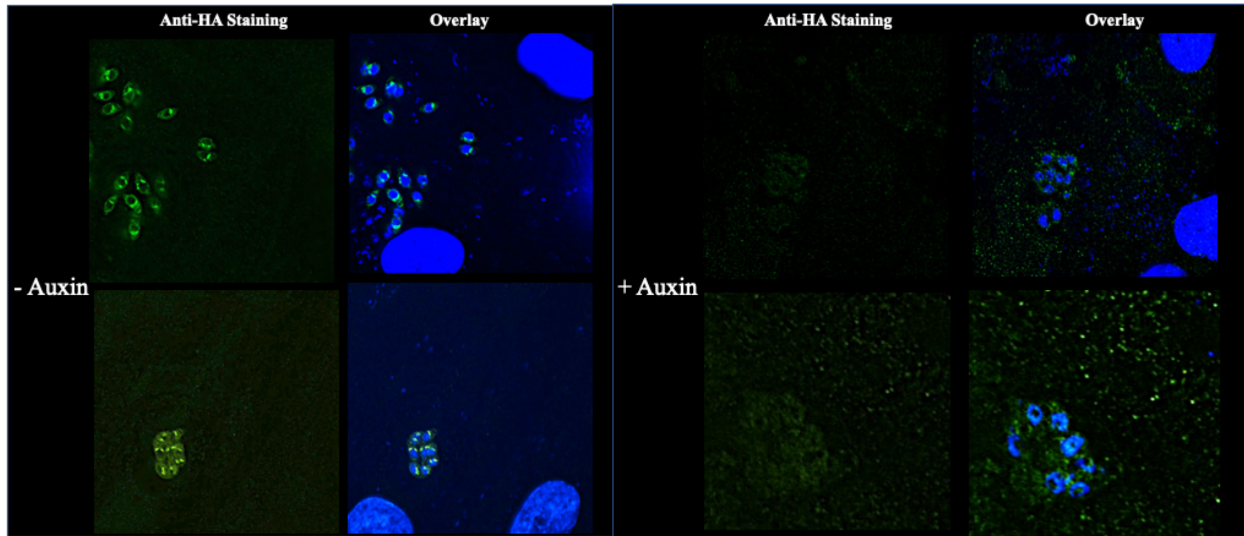
Intracellular Co-localization of TGGT1_253640
Figure 7: Co-localization experiment showing the HA tag in red, SERCA endoplasmic reticulum (ER) protein in green, overlay indicating partial colocalization.

TGGT1_253640 is downregulated by Auxin

Immunofluorescence Assay (IFA) confirmed the successful auxin induced downregulation of TGGT1_253640 in 4 hours (Fig. 8). IFA allows for the visualization of the POI using fluorescent staining. This visualization shows the time it takes for auxin to downregulate the POI. HFF cells on coverslips were infected with parasites and incubated with 250 uM of auxin. Three different time points (4, 6, and 12 hours) were used to determine the minimum needed auxin incubation time. Without auxin, (- auxin panels) the POI (anti-HA) is visible. The overlay adds a nuclear stain (DAPI in Blue). . If the downregulation of the POI is successful, the HA tag from the Immunofluorescence analysis will show no localization for the POI. After the addition of auxin, the POI (anti-HA stain), no longer is seen. This confirms the successful auxin induced knockdown of the POI and the functioning auxin-downregulation method in *T. gondii*. The induced downregulation was seen as early as 4 hours.

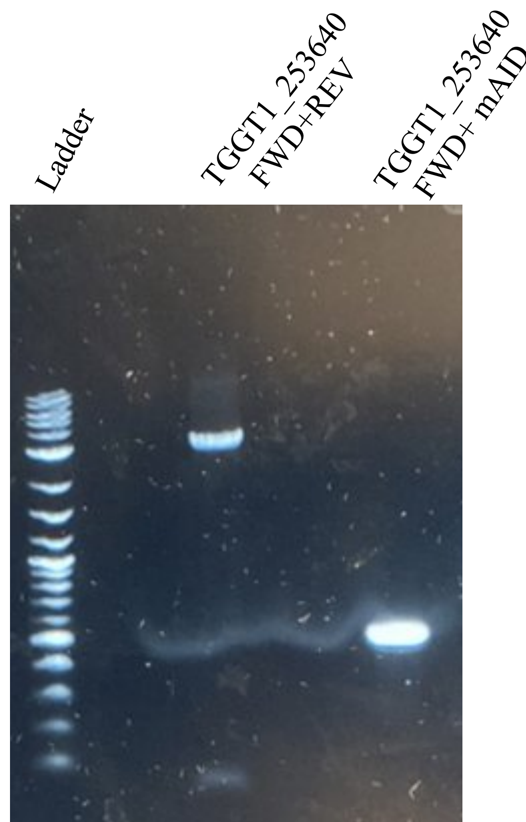
As discussed in preliminary results, drug selection was used to select for a (m)AID-3HA, Floxed HXGPRT construct. Parasites were exposed to mycophenolic acid (25 µg/ml) supplemented with xanthine (50 µg/ml) after 24 hours of drug selection. Clonal expression was verified using PCR screen to ensure that the mAID-HA cassette was still being expressed. This ensures that auxin induces the downregulation of TGGT1_253640. Fig.9 shows the desired modification. Lane 3 shows loss of original locus and Lane 5 shows proper integration of the

mAID-HA cassette.



Auxin induced downregulation of TGGT1_253640

Figure 8: Immunofluorescence of HA tag of 253540-mAID-HA (green) and DAPI (blue) showing downregulation of TGGT1_253640 by auxin as early as 4 hours.

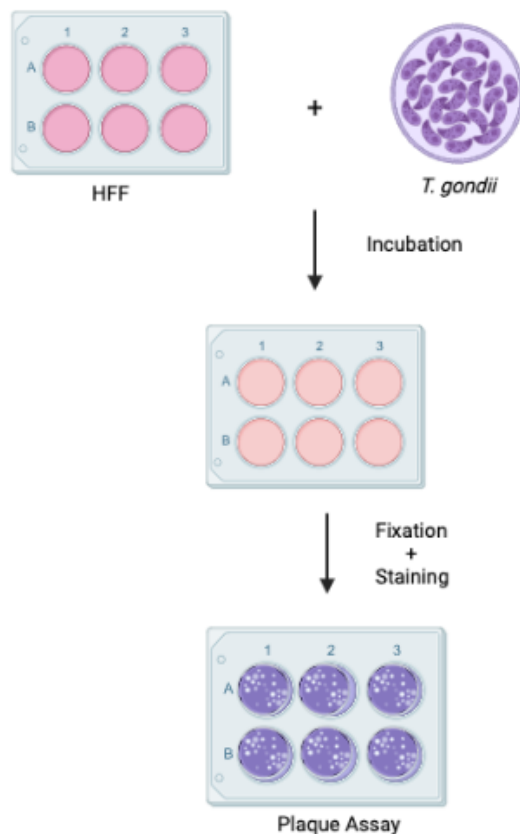


Successful Integration of the mAID-HA cassette
Figure 9: TGGT1_253640 FWD+REV and TGGT1_253640 FWD+ mAIDREV show the successful integration of the mAID-HA cassette.

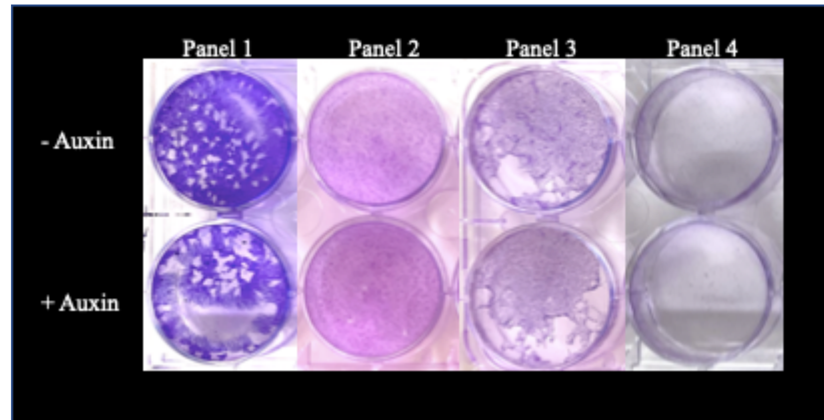
TGGT1_253640 importance for invasion, and egress of *T. gondii* needs to be studied further

Plaque assays were used to determine the role of TGGT1_253640 in *T. gondii* growth. Plaque assays were incubated for 7-10 days with and without auxin and conducted on control (TIR) and transgenic (TGGT1_253640) cells lines. The purpose of this assay was to determine how auxin induced downregulation of the POI affects the growth of *T. gondii*. If the POI is essential in *T. gondii* functioning, there would have been a significant difference in plaque size between wild type and transgenic parasites. The plaques, clearings due to host cell lysis, are visible against the crystal violet-stained host cells and represent the egress of *T.gondii* from the host cell after the parasite has engaged in a cycle of host attachment, invasion, and replication. The plaque assay showed that there are no noticeable growth defects associated with TGGT1_253640. Although the assay needs to be optimized to show a clear observable

phenotype; it appears that there is no likely difference between the plaques sizes and number formed between wildtype and transgenic parasites. A number of factors were optimized for the assay, including the determination of optimal parasite number (250-500 parasites / well), auxin concentration (250uM-500uM), and incubation time (7-10 days). Figure 11 shows plaque assay results from different trials. Panels one and three show the result of conducting a plaque assay without confluent host cells causing large clearings that are not the result of egress. These clearings are most likely due to older higher passage host cells that fail to adhere confluently to the culture dish. Panel two shows the resulting plaque assay when too little parasites are used/invaded, most likely due to parasites that were extracellular for too long; thus, lacking ability to invade host cells. Panel four shows the result of letting the parasites incubate past 7 days. The parasites egressed the whole host cell layer, so the crystal violet was not able to stain host cells to provide a contrast to see the plaques.



Plaque Assay Schematic
Figure 10: *T.gondii is* incubated in HFF host cells for 5-7 days before being fixed with ethanol and stained with crystal violet. The resulting clearings are from *T.gondii* lysing the host cells after egress. Figure made using BioRender.



Plaque Assay optimization
Figure 11: Plaque Assays of TGGT1_253640 parasites. A concertation of 250 uM of auxin was used to treat each cell line. Panel one had 500 parasites/well, panel two had too little parasites with 200 parasites/well, panel three had 500 parasites per well, and panel four with 500 parasites/well, grew for too long resulting in complete host cell lysis. Panels 1 and 3 had old host cells which resulted in the peeling of the host cell.

Discussion

This study was able to characterize the novel calcium regulator, TGGT1_253640. Through BLAST analysis the number of amino acids and the capacity for cation transport was confirmed. Protter visualization determined the presence of four transmembrane helices with intracellular N and C terminuses. IFA confirmed the successful auxin induced downregulation of TGGT1_253640 and demonstrated co-localization of the POI to the ER. While the ER is a major store of calcium, *T. gondii* contains other calcium stores including Golgi apparatus and mitochondria (Traina et al., 2018). It is possible that the POI localizes to the Golgi apparatus *T. gondii,* localization experiments using a Golgi apparatus marker will be conducted to confirm this prediction.

Further work needs to be done to analyze parasite fitness. This includes RFP (Red Fluorescence Protein) based growth and replication assays. Calcium assays and western blot will be conducted to further characterize the function of the proteins. RFP based growth and replication assays, parasite growth will measure parasite daily over a period of seven days by reading the fluorescence using a microplate reader using a 544-nm excitation and a 590-nm emission. Parasite growth in terms of average fluorescence (from quadruple wells) will be plotted against time to generate growth curves for parental induced vs. uninduced and transgenic parasite induced vs uninduced. The relative difference between induced and uninduced lines from the RPF assay will be assessed to infer whether POI contributes to parasite fitness. If the POI contributes to parasite fitness, then the growth curves for the induced lines will display reduced growth compared to the uninduced lines. We tried to generate stable RFP expressing TGGT1_253640mAID-HA parasites but in the absence of flow cytometer we were unable to recover stable parasites for this assay. Competition assays were designed to assess parasite fitness in direct comparison to wild type parasites and are currently ongoing to determine how the growth of parasites is affected by the auxin induced knockdown of gene. Briefly, equal number of transgenic and wild-type parasites are cocultured for several passages and incubated with 500 uM auxin or without auxin. After seven passages, wild type vs transgenic parasite numbers will be assessed by qPCR as described by Rosenberg et al. (2019). The parasites incubated with auxin are expected to experience slower growth compared to the parasites cultured without auxin if the gene plays any role in fitness of these parasites.

Calcium assays will be used to analyze the effects of the degraded protein on *T. gondii*. The calcium assay will be used to determine how the loss of the protein affects the parasite's calcium regulation. The assay will compare the calcium levels in a wildtype and transgenic

parasite's cytosol. If the predicted proteins are involved in essential calcium homeostasis in the parasite, we expect that upon downregulation with auxin, the transgenic parasites might demonstrate reduced ability to regulate calcium influx into the cytosol. Through calcium assays and RFP based assays, more information can be gathered on how TGGT1_253640 affects *T. gondii* and if it is essential for *T. gondii* functioning. Plaque assays determined that auxin induced downregulation of TGGT1_253640 resulted in no growth defects in *T. gondii.* Other experiments can be conducted before a conclusion about the role of TGGT1_253640 in *T. gondii* growth. This includes mouse survival experiments that can be used to analyze the importance of TGGT1_253640 in growth of *T. gondii*. A western blot is currently in process and will be used to further confirm the successful auxin-induced knockdown of TGGT1_253640. If the auxin-induced knockdown is successful, there will be no band present for the transgenic parasites cultured with auxin.

Additionally, further analysis is needed to determine the role TGGT1_222060 plays in *T.gondii* fitness. RFP based growth and replication assays, calcium assays, plaque assays, and western blot will be conducted on TGGT1_222060 to determine if the protein is essential in *T.gondii.*

The information gathered about TGGT1_253640 and TGGT1_222060 in this study contributes to the little-known information about calcium regulation in *T. gondii.* Many genes are predicted to be involved in calcium signaling, but the role of these calcium regulator genes is not known (Triana et al., 2018). Thus, the information collected in this study helps to further advance research into calcium signaling in *T. gondii.* Learning more about calcium regulator proteins and calcium signaling helps researchers gain more insight into potential drug targets that

can be used to create drugs that are effective against Toxoplasmosis and other disease-causing apicomplexan parasites.

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